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Research report

NMDA receptor antagonists attenuate the proconvulsant effect of juvenile social isolation in male mice

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ABSTRACT

Experiencing psychosocial stress in early life, such as social isolation stress (SIS), is known to have negative enduring effects on the development of the brain and behavior. In addition to anxiety and depressive-like behaviors, we previously showed that juvenile SIS increases susceptibility to pentylenetetrazole (PTZ)-induced seizures in mice through enhancing the nitrergic system activity in the hippocampus. In this study, we investigated the possible involvement of *N*-methyl-D-aspartate (NMDA) receptors in proconvulsant effects of juvenile SIS. Applying 4 weeks of SIS to juvenile male mice at postnatal day 21–23, we observed an increased susceptibility to PTZ as well as anxiety and depressive-like behaviors in adult mice. Intraperitoneal (i.p.) administration of NMDA receptor antagonists, MK-801 (0.05 mg/kg) and ketamine (0.5 mg/kg), reversed the proconvulsant effects of SIS in Isolated (and not social) housed animals. Co-administration of non-effective doses of nitric oxide synthase (NOS) inhibitors, 7NI (25 mg/kg) and L-NAME (10 mg/kg), with NMDA receptor antagonists, MK-801 (0.01 mg/kg) and ketamine (0.1 mg/kg) attenuated the proconvulsant effects of juvenile SIS only in isolated housed mice. Also, using real time RT-PCR, we showed that hippocampal upregulation of NR_{2B} subunit of NMDA receptor may play a critical role in proconvulsant effects of juvenile SIS by dysregulation of NMDA/NO pathway. In conclusion, results of present study revealed that experiencing SIS during adolescence predisposes the co-occurrence of seizure disorders with psychiatric comorbidities and also, alteration of NMDA receptor structure and function in hippocampus plays a role in proconvulsant effects of juvenile SIS through enhancing the NMDA/NO pathway.

1. Introduction

Social isolation, as a chronic stress, is a determinant psychosocial factor which has profound effects on the social functioning, quality of life and severity of disease in epileptic patients (Suurmeijer et al., 2001; McCagh et al., 2009). Although large body of evidence indicates that psychosocial stressors are accompanied by high morbidity and severity of disease in patients with epilepsy, there are few studies that have directly focused on the underlying mechanisms by which chronic stress increases seizure risk and occurrence (Yuen et al., 2007; Maguire and Salpekar, 2013). In this regard, clinical and preclinical data have shown that social isolation stress (SIS) increases the risk of seizure susceptibility in both human and

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rodents (Yuen et al., 2007; Chadda and Devaud, 2004; Matsumoto et al., 2003).

Experiencing juvenile SIS (as a valid animal model of chronic stress) has been reported to induce diversity of neurobehavioral and developmental changes in the brain (Fone and Porkess, 2008). Exposure to chronic stress mostly in the developmental stages of life is known to make remarkable neuroplastical alterations such as changes in receptors function and structure that affect excitatory and inhibitory synaptic neurotransmission in several regions of the brain (Maguire and Salpekar, 2013; Salzberg et al., 2007). In this context, *N*-methyl-D-aspartate (NMDA) receptors have gained special attention because of their involvement in the pathophysiology of a majority of brain diseases including seizure disorders (Kalia et al., 2008; Waxman and Lynch, 2005). It has been well documented that stress-induced increase in glutamatergic neurotransmission leads to excitotoxicity through activation of NMDA receptors and hence, causes brain injury (Olivenza et al., 2000; Ghasemi et al., 2014). Previous data showed that both NMDA receptor antagonists and nitric oxide synthase (NOS) inhibitors not only are able to reduce detrimental impacts of stress on the brain but also exerts anticonvulsant properties (Ghasemi and Schachter, 2011; Banach et al., 2011; Hardingham, 2009). On the other hand, juvenile SIS has been reported to alter excito-inhibitory balance by changing the regulation of NMDA receptors and nitrergic system in cortico-limbic areas (Petrovszki et al., 2013; Matsumoto et al., 2007; Zlatković et al., 2014). Surprisingly, underlying mechanisms by which SIS primes seizure vulnerability have not been well studied. We recently showed that experiencing SIS in the adolescence is associated with enhanced seizure susceptibility through activation of nitrergic system in the hippocampus (Amiri et al., 2014). Also, we previously demonstrated that nitrergic system and NMDA receptors contribute to development of anxiety and depressive like behaviors in adult mice following juvenile SIS (Amiri et al., 2015a; Haj-Mirzaian et al., 2015a).

In this study, we aimed to investigate whether NMDA receptors contribute to proconvulsant effect of juvenile SIS. Also, we investigated the possible interaction between the NMDA receptors and nitrergic system in the hippocampus of stressed animals to find a mechanism through which juvenile SIS exerts its negative effects.

2. Materials and methods

2.1. Animals

Male NMRI mice (Pasteur Institute, Tehran, Iran), weighing 10–12 g and in the postnatal state (PND: 21–23) were housed for 4 weeks under two different conditions: (1) social condition (SC) and (2) isolated condition (IC). Socially conditioned animals were housed in groups (6 mice per cage: $25 \times 25 \times 15$ cm) while IC mice were housed individually in Plexiglas boxes ($24 \times 17 \times 12$ cm) under standard laboratory conditions (free access to food and water, temperature: 22 ± 2 °C, humidity: 50 ± 10% and 12-h light–dark cycle). All procedures in this work were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) and institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, TUMS).

2.2. Forced swimming test (FST)

We used FST as a behavioral test in order to evaluate the effects of juvenile SIS on behavioral despair of animals (Haj-Mirzaian et al., 2014; Russo et al., 2013; Cryan and Holmes, 2005). In brief, mice were individually placed in an open glass cylinder (diameter: 10 cm, height: 25 cm) containing 19 cm water (23 ± 1 °C). Mice were allowed to swim for 6 min, and the immobility time was recorded during the last 4 min of the test. Immobility behavior was considered when the animal remained floating motionless in the water and made only those necessary movements to keep its head above water.

2.3. Splash test

Splash test was used in order to investigate the possible effect of SIS on behaviors related to motivational and self-care difficulties in animals. In this test, grooming behavior of mice, which can be considered as an indirect measure of palatable solution intake was measured. A 10% sucrose solution was squirted on the dorsal coat of each animal in the home cage. In case of SC mice, animals were housed individually for 24 h before the test. Behaviors of mice were videotaped for 5 min and grooming activity time including nose/face grooming, head washing, and body grooming were scored by an experimenter who was blind to the treatment conditions (Haj-Mirzaian et al., 2015b; Nollet et al., 2013).

2.4. Elevated plus maze (EPM)

This test was used in order to evaluate the effects of SIS on anxiety-like behaviors in mice (Amiri et al., 2015b). The EPM apparatus was made of black opaque Plexiglas and consisted of two open (30×5 cm) and closed ($30 \times 5 \times 15$ cm) arms, which were connected by a platform area (5×5 cm). Testing room was dimly illuminated and animals were individually placed in the center of the EPM facing to closed arm and each behavioral session was videotaped for a 5 min period. The total time spent in the open arms as well as number of entries into the open arms were recorded over a period of 5 min and reported as percentages (Table 1).

2.5. Drugs and treatments

The following drugs were used in this study: pentylenetetrazole (PTZ), NG-nitro-L-arginine methyl ester (L-NAME), 7-nitroindazole (7NI), MK-801, and ketamine. Drugs were purchased from Sigma (UK) and were dissolved in saline (except for 7-NI which was suspended in Tween80 1%) and were administered in the volume of 5 ml/kg animal weights. In order to investigate the seizure susceptibility in experimental animals, we administered PTZ intravenously (0.5%, i.v.) and all other drugs intraperitoneally (i.p.).

2.6. Determination of clonic and tonic seizure threshold

In order to measure SIS-induced alterations in the seizure susceptibility, we assessed both clonic and tonic seizure thresholds in mice using the method that was previously described (Amiri et al., 2014; Amini-Khoei et al., 2015) with little modifications. Briefly, a winged infusion set (30 gauge) was used to infuse the PTZ (0.5%) at a constant rate of 1 ml/min (using NE 1000, New Era Pump System, Inc.) into the tail vein of the freely moving subjects. Each animal behavior was videotaped by a camera and observed seizure signs were reported as (1) onset of clonus seizures (clonus threshold) characterized by forelimbs clonus (following by full clonus of the body and loss of righting ability) and (2) tonic hind limb extension (tonic threshold). Infusion was halted at the appearance of tonic hind limb extension, characterized by extreme muscle rigidity starting at the head and progressing along the body with full extended hind limbs finally. The minimal dose of PTZ (mg/kg mouse weight) required for inducing seizure signs was considered as the indices of both seizure thresholds. As such, seizure threshold is dependent on the dose and time of PTZ administration.

Table 1
Experimental groups of the study: housing conditions (start and endpoint from PND), treatments, and number of animals in each experimental group were illustrated.

Groups	Conditions (PND start-end)	Treatments	Numb.	Behavioral tests (PND time)	Molecular tests
SC1	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	8–9	EPM (Khan et al., 2015) & FST (Jones et al., 2014)	–
SC2	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	8	Splash test (Jones et al., 2014)	–
SC3	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC4	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Saline (5 ml/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC5	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Tween80 (5 ml/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC6	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Ketamine (0.1 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC7	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Ketamine (0.5 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC8	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	MK-801 (0.01 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–

Table 1 (*Continued*)

Groups	Conditions (PND start-end)	Treatments	Numb.	Behavioral tests (PND time)	Molecular tests
SC9	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	MK-801 (0.05 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC10	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC11	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	7-NI (15 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC12	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + Ketamine (0.1 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC13	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + MK-801 (0.01 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC14	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + Ketamine (0.1 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC15	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + MK-801 (0.01 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
SC16	4 weeks of SC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	3	–	NR_{2A} and NR_{2B} gene expression

Table 1 (*Continued*)

Groups	Conditions (PND start-end)	Treatments	Numb.	Behavioral tests (PND time)	Molecular tests
IC1	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	8–9	EPM (Khan et al., 2015) & FST (Jones et al., 2014)	–
IC2	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	8	Splash test (Jones et al., 2014)	–
IC3	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC4	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Saline (5 ml/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC5	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Tween80 (5 ml/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC6	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Ketamine (0.1 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC7	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	Ketamine (0.5 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC8	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	MK-801 (0.01 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–

Table 1 (*Continued*)

Groups	Conditions (PND start-end)	Treatments	Numb.	Behavioral tests (PND time)	Molecular tests
IC9	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	MK-801 (0.05 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC10	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC11	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	7-NI (15 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC12	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + Ketamine (0.1 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC13	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + MK-801 (0.01 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC14	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + Ketamine (0.1 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC15	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	L-NAME (10 mg/kg) + MK-801 (0.01 mg/kg)	6–8	PTZ-seizure model (Jones et al., 2014)	–
IC16	4 weeks of IC (Haj-Mirzaian et al., 2015a,b, 2014; Russo et al., 2013; Cryan and Holmes, 2005; Nollet et al., 2013; Amiri et al., 2015b; Amini-Khoei et al., 2015; Schmittgen and Livak, 2008; Blanchard et al., 2001; MacKenzie and Maguire, 2015; Lupien et al., 2009; Huang, 2014; Andersen and Teicher, 2008; Žiburkus et al., 2013; Zhao et al., 2009; Gan et al., 2014; Frasca et al., 2011; Fujikawa, 2005; Chang et al., 2015; Toua et al., 2010; Kawasaki et al., 2011; Turnock Jones et al., 2009; Ewald et al., 2008; Di Maio et al., 2013; Forder and Tymianski, 2009; Watanabe et al., 2013; Przegaliński et al., 1996; Sattler et al., 1999; Khan et al., 2015)	–	3	–	NR_{2A} and NR_{2B} gene expression

Table 2
Primers.

Gene	Sequence (5′→3′) Forward	Reverse
grin2a	ACGTGACAGAACGCGAACTT	TCAGTGCGGTTCATCAATAACG
grin2b	CAGCAAAGCTCGTTCCCAAAA	GTCAGTCTCGTTCATGGCTAC
Hprt1	TGCTCGAGATGTGATGAAGG	AAGCAGATGGCCACAGAACT

2.7. Real-time RT-PCR analysis for NMDA receptor subunits

Animals were sacrificed under mild anesthesia and hippocampi were dissected on ice-cold surface and immediately homogenized with Trizol (Invitrogen) in order to isolate the total RNA from the samples. Changes in mRNA levels of desired genes were measured by qRT-PCR after reverse transcription of 1 μg of RNA from each sample using PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). qRT-PCR was performed on a light cycler instrument (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). Thermal cycling conditions involved an initial activation step for 30 s at 95 °C followed by 45 cycles including a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was applied to validate whether all primers yielded a single PCR product. The primers used for NR2A and NR2B genes expression (grin2a and grin2b) are listed in Table 2. Hypoxanthine phosphoribosyl transferase1 (hprt1) was amplified as normalizer and the fold change in expression of each target mRNA relative to hprt1 was calculated on the basis of $2-\Delta\Delta ct$ relative expression formulas (Schmittgen and Livak, 2008).

2.8. Experimental design

To determine whether juvenile SIS was able to provoke behavioral abnormalities in adult mice, both SC and IC mice were subjected to the behavioral tests including FST, splash test and EPM, after 4 weeks exposure to different housing conditions. Also, we evaluated the possible effect of SIS on clonic and tonic seizure threshold using intravenous PTZ-induced seizures in animals. In another experimental design, we investigated the possible effect of NMDA receptor antagonists on proconvulsant effect of SIS. In this regard, we treated both SC and IC mice with ketamine (0.1 and 0.5 mg/kg, i.p., 60 min before PTZ seizure test) and MK-801 (0.01 and 0.05 mg/kg, i.p., 60 min before PTZ seizure test).

In the next step, we evaluated the possible role of nitrergic system on the anti/proconvulsant effect of NMDA receptor antagonists. In this regard, we treated both SC and IC mice with non-effective doses of NMDA antagonists which were obtained from the last parts of the study and either of the following drugs: L-NAME (10 mg/kg, i.p., 45 min before PTZ seizure test) and 7-NI (15 mg/kg, i.p., 45 min before PTZ seizure test). Doses of each drug were chosen according to the pilot treatments which were published in our previous studies (Amiri et al., 2014). To exclude the possible effect of vehicle administration, SC and IC groups were injected with 5 ml/kg sterile physiological saline and Tween80 1%. We also investigated the NR_{2A} and NR_{2B} genes expression (Nr3c1 and Nr3c2) levels in the hippocampus of SC and IC groups. Each experimental animal group consists of 6–9 in behavioral assessments, and 3 in molecular evaluation. Experimental design of the study consisting of housing conditions, age, and number of animals in each experiment, are summarized in Table 2.

2.9. Statistical analysis

Comparison between the groups was analyzed using *t*-test and one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered statistically significant. The factors were housing [social condition (SC) and isolation condition (IC)] and treatments [control: no treatment and treatment: drug-administered animals] for all assessments.

3. Results

3.1. Effects of juvenile SIS on mice behavior

In order to validate the applied SIS paradigm, SC and IC animals were evaluated using behavioral tests including FST, splash test, and EPM. Social isolation stress increased the immobility time in IC mice compared with SC animals in the FST ($t=5.10$, $df=14$, $P<0.001$, Fig. 1a). In the splash test, SIS induced a significant decline in grooming activity time in IC mice in comparison with SC mice ($t=6.964$, $df=14$, $P<0.001$, Fig. 1b). In the EPM, percentage of spent time in the open arms and percentage of open arms entries were evaluated as variables relevant to anxiety-like behaviors. In comparison with SC mice, SIS remarkably decreased percentage of spent time in the open arms ($t=3.124$, $df=14$, $P<0.01$, Fig. 1c) as well as percentage of open arms entries ($t=4.598$, $df=16$, $P<0.001$, Fig. 1d) in IC mice.

3.2. Juvenile SIS reduced clonic and tonic seizure thresholds in PTZ model of seizures

Using PTZ model of seizures, SIS significantly decreased the seizure thresholds in IC mice. Data have revealed a significant difference between IC and SC animals in relation to both clonic ($t=9.066$, $df=14$, $P<0.001$, Fig. 2a) and tonic ($t=7.80$, $df=10$, $P<0.001$, Fig. 2b) seizure thresholds.

3.3. NMDA receptor antagonists reverse the proconvulsant effect of juvenile SIS

Clonic and tonic seizure thresholds were determined for the different doses of NMDA antagonists including MK-801 and ketamine in SC and IC mice. ANOVA analysis showed that there were significant differences between all treated groups in clonic ($F(11, 84)=11.11$, $P<0.001$, Fig. 3a) and tonic ($F(11, 60)=20.80$, $P<0.001$, Fig. 3b) seizure thresholds. Fig. 3 presents significant differences in clonic and tonic seizure thresholds between IC and SC groups as well as saline, MK-801 (0.01 mg/kg), and ketamine (0.1 mg/kg) treated groups (***$P<0.001$ for all). However, there was no significant difference between SC and IC animals that were administered with MK-801 (0.05 mg/kg), and ketamine (0.5 mg/kg) in both clonic and tonic seizure thresholds ($P>0.05$).

Tukey's test showed that injection of ketamine (0.5 mg/kg) significantly reversed the proconvulsant effect of IC mice when compared with IC saline-injected group in the both clonic (##$P<0.01$) and tonic (###$P<0.001$) seizure thresholds. However, administration the lower doses of ketamine (0.1 mg/kg) had no significant effect on the clonic and tonic seizure thresholds in IC mice ($P>0.05$). On the other hand, data have shown that MK-801 (0.05 mg/kg) significantly increased the clonic and tonic seizure thresholds in IC mice when compared with saline treated IC groups in the both clonic (##$P<0.01$) and tonic (#$P<0.05$) seizure models. However, lower dose of MK-801 (0.01 mg/kg) did not alter the clonic and tonic seizure threshold in IC animals ($P>0.05$). Also, none of these treatments affected the clonic and tonice seizure thresholds in SC mice ($P>0.05$).

3.4. Nitrergic system contributes to anticonvulsant effect of NMDA receptor antagonists in socially isolated animals

In the first part we try to investigate the possible effect of L-NAME (non-selective NOS inhibitor) on the anticonvulsat effect

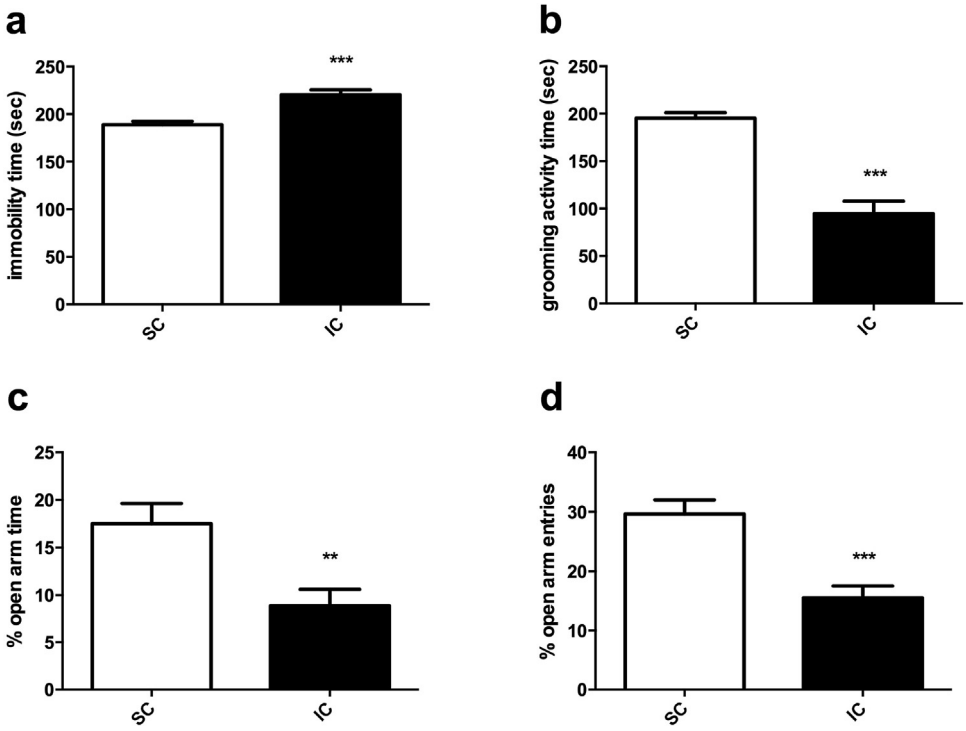

Fig 1. Effect of different housing conditions, social condition (SC) and isolated condition (IC), on the immobility time in the FST ($n = 8$) (a), grooming activity time in the splash test ($n = 8$) (b), percentage of time spent in the open arm in the EPM ($n = 8$) (c), and percentage of open arm entries in the EPM ($n = 9$) (d). Values are expressed as the mean ± S.E.M. from 8 animals and were analyzed using *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the SC group.

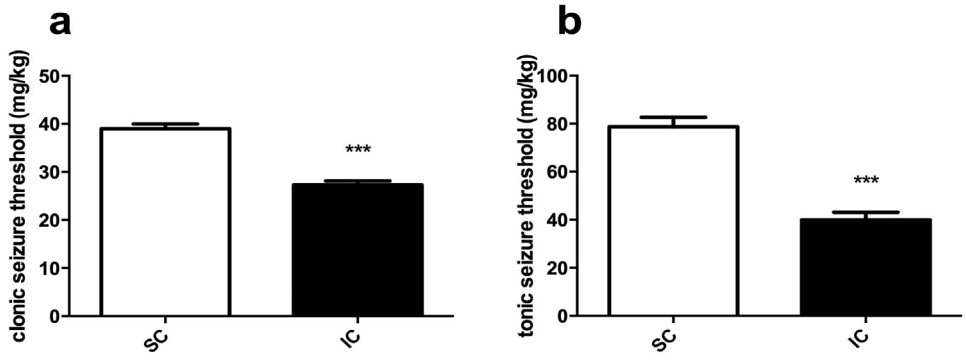

Fig. 2. Effect of different housing conditions, social condition (SC) and isolated condition (IC), on the colonic seizure ($n = 8$) (a), and tonic seizure ($n = 6$) (b) thresholds in the PTZ-model of seizure. Values are expressed as the mean ± S.E.M. from 8 animals and were analyzed using *t*-test. $^{***}P < 0.001$ compared with the SC group.

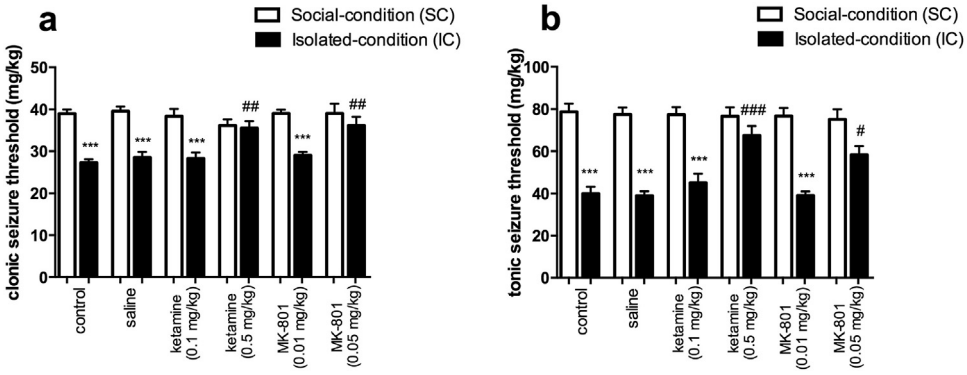

Fig. 3. Effects of different housing conditions (social condition (SC) and isolated condition (IC)) and treatments; including saline, ketamine (0.1 and 0.5 mg/kg), MK-801 (0.01 and 0.05 mg/kg) on the clonic ($n = 8$) (a), and tonic ($n = 6$) (b) seizure thresholds in the PTZ-model of seizure. Values are expressed as the mean ± S.E.M. and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{***}P < 0.001$ compared with the SC saline treated group. $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ compared with the IC saline treated group.

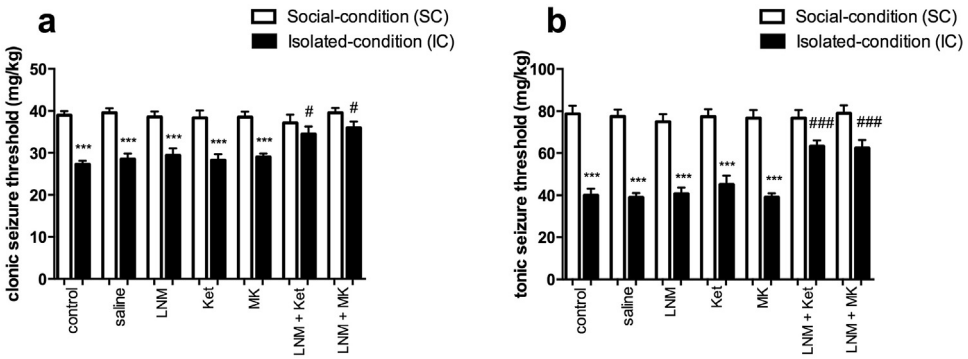

Fig. 4. Effects of different housing conditions (social condition (SC) and isolated condition (IC)) and treatments; including saline, L-NAME (LNM)(10 mg/kg), ketamine (Ket)(0.1 mg/kg), MK-801 (MK)(0.01 mg/kg), and L-NAME (10 mg/kg) + ketamine (0.1 mg/kg)/MK-801 (0.01 mg/kg) co-treatments on the clonic ($n = 8$) (a), and tonic ($n = 6$) (b) seizure thresholds in the PTZ-model of seizure. Values are expressed as the mean ± S.E.M. and were analyzed using one-way ANOVA followed by Tukey's post hoc test. ***$P < 0.001$ compared with the SC saline treated group. #$P < 0.05$ and ###$P < 0.001$ compared with the IC saline treated group.

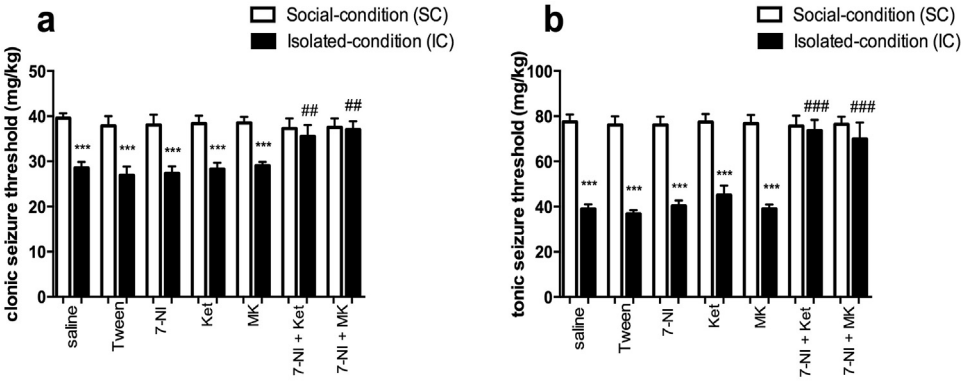

Fig. 5. Effects of different housing conditions (social condition (SC) and isolated condition (IC)) and treatments; including saline, Tween80 1%, 7-NI (15 mg/kg), ketamine (Ket) (0.1 mg/kg), MK-801 (MK) (0.01 mg/kg), and 7-NI (15 mg/kg) + ketamine (0.1 mg/kg)/MK-801 (0.01 mg/kg) co-treatments on the clonic ($n = 8$) (a), and tonic ($n = 6$) (b) seizure thresholds in the PTZ-model of seizure. Values are expressed as the mean ± S.E.M. and were analyzed using one-way ANOVA followed by Tukey's post hoc test. ***$P < 0.001$ compared with the SC saline treated group. ##$P < 0.01$ and ###$P < 0.001$ compared with the IC saline treated group.

of NMDA antagonists in IC animals. One-way ANOVA analysis has shown that there are significant differences between the groups in the clonic ($F(13, 96) = 11.80$, $P < 0.001$, Fig. 4a) and tonic ($F(13, 70) = 25.63$, $P < 0.0001$, Fig. 4b) seizure models. Fig. 4 indicates that there were significant differences in the clonic and tonic seizure thresholds between IC and SC controls as well as saline, L-NAME (10 mg/kg), ketamine (0.1 mg/kg), and MK-801 (0.01 mg/kg) treated groups (***$P < 0.001$ for all groups). However, there was no any significant difference between SC and IC groups when animals were co-treated with L-NAME (10 mg/kg) and ketamine (0.1 mg/kg)/MK-801 (0.01 mg/kg) ($P > 0.05$).

Tukey's test showed that co-administration of L-NAME and Ketamine (#$P < 0.05$ and ###$P < 0.001$) as well as co-treatment of L-NAME and MK-801 (#$P < 0.05$ and ###$P < 0.001$) significantly improved the both clonic and tonic seizure thresholds of IC mice when compared to IC saline-treated group. None of these treatments affected the clonic and tonic seizure thresholds in SC mice ($P > 0.05$).

In the next part, we evaluated the possible effect of 7-NI (selective nNOS inhibitor) on the anticonvulst effect of NMDA antagonists. One-way ANOVA analysis shows that there were significant differences between the groups in the clonic ($F(16, 98) = 7.522$, $P < 0.001$, Fig. 5a) and tonic ($F(13, 70) = 21.07$, $P < 0.001$, Fig. 5b) seizure thresholds. There were significant differences in the clonic and tonic seizure thresholds between IC and SC controls as well as saline, Tween80, 7-NI (15 mg/kg), ketamine (0.1 mg/kg), and MK-801 (0.01 mg/kg) treated groups (***$P < 0.001$ for all groups). However, there was no significant difference between SC and IC animals in 7-NI (10 mg/kg) and ketamine (0.1 mg/kg)/MK-801 (0.01 mg/kg) co-administered groups ($P > 0.05$).

Tukey's test showed that co-injection of 7-NI and Ketamine (##$P < 0.01$ and ###$P < 0.001$) as well as co-treatment of 7-NI and MK-801 (##$P < 0.01$ and ###$P < 0.001$) significantly enhanced the clonic and tonic seizure thresholds of IC mice when compared to IC Tween80/saline-treated group. None of these treatments affected the clonic and tonic seizure thresholds in SC mice ($P > 0.05$).

3.5. *Juvenile SIS up regulates the NR_{2B} but not NR_{2A} subunit of NMDA receptor in the hippocampus*

Fig. 6 shows the effects of juvenile SIS on expression of NMDA receptors genes. *t*-test analysis demonstrated the over-expression of NR_{2B} ($t = 22.38$, df = 4, $P < 0.001$) in hippocampus of IC mice in comparison with SC animals. However, no significant change in expression of NR_{2A} was observed in IC mice when compared to SC mice ($t = 0.4554$, $df = 4$, $P > 0.05$). In addition, there was no significant effect of housing conditions (SC and IC) on hprt1 expression.

4. Discussion

Results of the current study showed that experiencing SIS in the early adolescence increased seizure susceptibility to PTZ and also, altered NMDA receptor structure in the hippocampus. We demonstrated that increase in the seizure vulnerability was associated to alterations in NMDA/NO regulation in the hippocampus of IC animals. Unlike SC mice, administration of non-effective doses of

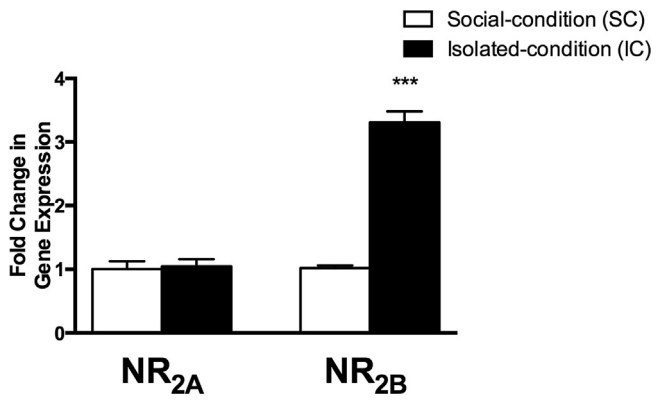

Fig. 6. Effect of different housing conditions, social condition (SC) and isolated condition (IC) on NMDA receptor subunits (NR_{2A} and NR_{2B}) gene expression in the hippocampus of animals. Values are expressed as the mean ± S.E.M. and were analyzed using *t*-test. ***$P < 0.001$ compared with the SC control group in each coupled columns.

NMDA receptor antagonists (ketamine and MK-801) reversed the proconvulsant effects of SIS in IC mice. We found that increased expression of NMDA receptor subunit NR_{2B} following 4 weeks of SIS may account for increased seizure susceptibility to PTZ by enhancing the NMDA/NO pathway in the hippocampus.

Applying animal models of chronic stress has been suggested as useful tools to investigate the underlying mechanisms through which, stress exerts its detrimental effects on the brain and behavior (Blanchard et al., 2001). Although there are recent studies which have focused on the influence of chronic stress on seizure activity (Salzberg et al., 2007; Russo et al., 2013; MacKenzie and Maguire, 2015) underpinning mechanisms by which social environment affects the seizure susceptibility have not been well studied. Accumulating evidence indicates that exposure to early life stress during developmental stages of brain engages in shaping of vast majority of brain disorders including seizures (Lupien et al., 2009; Huang, 2014). In this context, juvenile SIS has been shown to induce long lasting cellular and molecular alterations in development of cortico-limbic regions (Andersen and Teicher, 2008) and primes seizure occurrence in adulthood (Matsumoto et al., 2003; Amiri et al., 2014). Several lines of research indicate that imbalance between excitatory and inhibitory neurotransmission contributes to pathophysiology of seizure disorders (Žiburkus et al., 2013). Our results showed that juvenile SIS-induced seizure susceptibility to PTZ accompanied with anxiety and depressive-like behaviors. These results are consistent with previous studies which have reported that adolescent SIS not only is able to render seizure occurrence but also, provokes anxiety and depressive-like behaviors in adult animals (Chadda and Devaud, 2004; Matsumoto et al., 2003; Amiri et al., 2014). We recently demonstrated that blockade of NMDA receptors by subeffective doses of ketamine and MK-801 could reverse the depressive-like behaviors following adolescent SIS (Salzberg et al., 2007; Haj-Mirzaian et al., 2015a). Previous studies have reported that SIS negatively makes changes in various neurotransmission systems including both nitrergic and glutamatergic systems in the brain (Zhao et al., 2009; Gan et al., 2014). Furthermore, a recent study by Frasca et al., (2011) has revealed that misplacement of NMDA receptors in the limbic area provokes excitatory state and epileptogenesis. Administration of NMDA receptor antagonists has been reported to have potent anticonvulsant properties in the literature (Ghasemi and Schachter, 2011; Fujikawa, 2005). In consistence with previous reports, treating IC mice with subeffective doses of both ketamine and MK-801, as non-comparative NMDA receptor antagonists, increased both clonic and tonic seizure thresholds in IC animals. However, same treatments to socially housed counterparts did not change the seizure threshold.

Previous research has shown that SIS increases the NMDA receptors in the hippocampus (Chang et al., 2015) and NMDA receptor binding capacity in the frontal cortex (Toua et al., 2010). Also, metabotropic glutamate receptors (mGluRs) were reported to undergo functional changes in socially isolated animals (Kawasaki et al., 2011). Moreover, up-regulation of NMDA receptor subunits such as NR_{2A} in the prefrontal cortex (Turnock Jones et al., 2009) along with NR_{2A} and NR_{2B} in the hippocampus (Zhao et al., 2009) was reported in previous studies. Thus, it is possible that abnormal activity of glutamatergic system plays a part in the proconvulsant effect of juvenile SIS. To support this, we found that juvenile SIS increased expression of NR_{2B} (but not NR_{2A}) subunit of NMDA receptor in the hippocampus. Our results were in line with recent findings demonstrating that SIS increases the expression of NR_{2A} and NR_{2B} subunits of NMDA receptors in the different regions of the brain such as hippocampus and cortical areas (Chang et al., 2015; Turnock Jones et al., 2009). It has been well documented that NR_{2B} subunit of NMDA receptor is potently involved in several (patho) physiological processes such as developmental plasticity and epilepsy (Frasca et al., 2011; Ewald et al., 2008; Di Maio et al., 2013). In this context, ample evidence indicates that NR_{2B} subunit of NMDA receptor is responsible for massive calcium influx into postsynaptic hippocampal neurons that results in hyper-excitability and activation of diversity of biological pathways including nitrergic system (Forder and Tymianski, 2009). These results indicate that NR2B NMDA receptor subunit is involved in the proconvulsant effect of juvenile SIS in IC mice. Large body of evidence suggests that nitrergic system participates in the pathophysiology of seizure disorders (Watanabe et al., 2013; Przegaliński et al., 1996). Also, there are pieces of evidence indicating that inhibition of NOS (mostly nNOS) by NOS inhibitors such as L-NAME and 7NI exerted anticonvulsant effects in experimental studies (Banach et al., 2011). It is well accepted that activation of NMDA receptors is associated with an increase in the NO production through activation of nNOS (Sattler et al., 1999). Recently, we showed that juvenile SIS-induced increase in hippocampal NO was correlated with an increase in seizure susceptibility to PTZ. In this regard, results of this study showed that co-administration of subeffective doses of NMDA receptor antagonists (ketamine and MK-801) along with L-NAME or 7NI potently reversed the proconvulsant effect of SIS by increasing both clonic and tonic seizure thresholds in IC mice. However, such effects were not observed in SC mice treated with 7NI or L-NAME alone or in combination with NMDA receptor antagonists. These results suggest that enhanced seizure susceptibility to PTZ in IC mice is partially related to enhanced activation of NMDA/NO pathway mainly through interaction of NR_{2B} and nNOS. Also, these changes in the structure and function of NMDA receptors have been shown to have negative effects on emotional state of mice (Chang et al., 2015). However, further studies are needed to determine whether other neurotransmission abnormalities are involved in the proconvulsant effect of SIS. For instance, it has been reported that opioidergic and GABAergic system may be involved in the proconvulsant effect of post weaning SIS (Matsumoto et al., 2003; Khan et al., 2015). Considering that experiencing chronic psychosocial stress during adolescence is regarded as a serious risk for development of mental difficulties in females (Andersen and Teicher, 2008), effects of juvenile SIS on developing brain and initiation of seizure disorders have not been well studied in female animals (Jones et al., 2014).

In conclusion, results of our study showed that behavioral abnormalities following juvenile SIS are accompanied by increased susceptibility to PTZ-induced seizures and, dysfunction of NMDA/NO pathway through NR_{2B} overexpression may play a

role in the negative impact of SIS on the both tonic and clonic seizure thresholds.

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